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(54) Title: NOVEL PROTEINS AND CLONED GENES FOR DIAGNOSIS AND PROPHYLAXIS OF BABESIOSIS (57) Abstract <p>The subject invention concerns the identification of novel merozoite surface proteins of <i>Babesia bovis</i>. Also disclosed are monoclonal antibodies to these proteins as well genes which encode for the proteins. The invention further concerns the use of the novel proteins, recombinant DNA clones, and monoclonal antibodies in the detection, treatment, and prophylaxis of babesiosis.</p> <p style="text-align: right;"> USSN: To be assigned Applicant: Erik DE VRIES, et al. Express Mail No: EV 630723128 US </p>		

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DESCRIPTIONNOVEL PROTEINS AND CLONED GENES
FOR DIAGNOSIS AND PROPHYLAXIS OF BABESIOSISCross-Reference to a Related Application

This application is a continuation-in-part of our co-pending application Serial No. 333,155, filed April 4, 1989.

Background of the Invention

Bovine babesiosis is a tick-transmitted, hemoparasitic disease caused by intraerythrocytic protozoa belonging to the genus Babesia. The disease caused by Babesia manifests itself clinically by fever and extensive hemolytic anemia that often leads to hypotensive shock, cerebral involvement, and death. More than a half billion cattle are estimated to be at risk of acquiring babesiosis. This disease represents a primary impediment to food and fiber production in much of the world.

To date, control of bovine babesiosis in enzootic areas has been partially successful through vaccination with attenuated strains of Babesia spp. or with more virulent strains followed by chemotherapeutic control. Protective immunity in babesiosis may be directed against one or more surface antigens associated with sporozoites, infected erythrocytes, and/or merozoites. Merozoite surface antigens are important in the pathogenesis and immunology of babesiosis due to their role in the parasite's recognition of, attachment to, and penetration of host erythrocytes and their accessibility to the immune system.

Recently, progress has been made toward the identification and characterization of specific immunogens of merozoites of Babesia bovis (Smith, R.D., M.A. James, M. Ristic, M. Aikawa, and C.A. Vega Y Murgula [1981] Science 212:335-338; Wright, I.G., B.V. Goodger, K. Rode-Bramanis, J.S. Matlick, D.F. Mahoney, and D.J. Waltisbuhl [1983] Z. Parasitenkd. 69:703-714; Wright, I.G., G.B.

Mirre, K. Rode-Bramanis, M. Chamberlain, B.V. Goodger, and D.J. Mahoney [1985] Infect. Immun. 48:109-113; Commins, M.A., B.V. Goodger, and I.G. Wright [1985] Int. J. Parasitol. 15:491-495; Wright, I.G. and P.W. Riddles [1986] "Biotechnological Control of Tick-Borne Disease," Meeting of the Food and Agriculture Organization of the United Nations, 6-10 October 1986, pp. 1-21, Rome, Italy; Waltisbuhl, D.J., B.V. Goodger, I.G. Wright, G.B. Mirre, and M.A. Commins [1987] Parasitol Res. 73:319-323; Goff, W.L., W.C. Davis, G.H. Palmer, and T.C. McGuire [1988] Infect. Immun. 56:2363-2368) and Babesia bigemina (McElwain, T.F., L.F. Perryman, W.C. Davis, and T.C. McGuire [1987] J. Immunol. 138(7):2298-2304). However, in only one instance (Smith et al., 1981) were antigens which provided protection against infection determined to be surface-exposed on merozoites as opposed to cytoplasmic in location.

Bovine babesiosis can be caused by either Babesia bigemina or Babesia bovis. These parasites have antigenic similarities and differences that may have important functional roles in the induction of protective immunity and antibody-based diagnosis. Also, B. bovis isolates, including the current Australian vaccine strain, are now known to consist of subpopulations that vary antigenically, in virulence, and in abundance within an isolate (Cowman, A.F., P. Timms, and D.J. Kemp [1984] Mol. Biochem. Parasitol. 11:91-103; Gill, A.C., A.F. Cowman, N.P. Stewart, D.J. Kemp, and P. Timms [1987] Exp. Parasitol. 63:180-188).

Current vaccine strategies include the use of attenuated live Babesia bovis parasites and various inactivated preparations (Montenegro-James, S., M. Toro Benitez, E. Leon, R. Lopez, and M. Ristic [1987] Parasitol. Res. 74:142-150; Smith et al., 1981; U.S. Patent No. 4,762,711 issued to Buening et al.; Kuttler, K.L., M.G. Levy, M.A. James, M. Ristic [1982] Am. J. Vet. Res. 43(2):281-284). The attenuated vaccine provides the best protection against challenge with both homologous and heterologous strains, although there are a number of serious disadvantages, including a short shelf-life, variation in virulence, contamination with host erythrocyte stroma, and perpetuation of the life cycle by creation of a carrier state. Inactivated vaccines induce protection against challenge with homologous

strains; however, only partial protection occurs against challenge with heterologous strains.

Animals that survive natural field infection or that recover from infection with an attenuated vaccine strain are protected against clinical disease. However, premunization in this manner is expensive, impractical in developing countries that lack the necessary infrastructure, and a potential mode of transmission for other blood-borne diseases.

Brief Summary of the Invention

Disclosed and claimed here are novel merozoite proteins of Babesia bovis. These proteins are known to be expressed on the surface of the merozoite and may be used to raise neutralizing antibodies. Thus, they can be used in the formulation of subunit vaccines for the prophylaxis of bovine babesiosis. Several of the proteins described here raise antibodies to both Babesia bovis and Babesia bigemina, while others are species, or even isolate, specific.

Also disclosed are monoclonal antibodies to bovine babesiosis antigens. These monoclonal antibodies are used to identify merozoite surface antigens and may be used in the treatment and/or diagnosis of bovine babesiosis.

A further element of the invention is the identification of genes which code for Babesia proteins. These genes can be used to make recombinant proteins which can be utilized for vaccines.

The invention also provides a means of detecting the presence of disease-causing Babesia organisms. The detection method involves the use of DNA probes which selectively identify the presence of these organisms.

Brief Description of the Drawings

Figure 1 is the translated DNA and amino acid sequence of lambda-Bo44.

Figure 2 is the DNA and amino acid sequence for rBv42.

Figure 3 is the DNA sequence for rBv60.

Figure 4 is the amino acid sequence for rBv60.

Detailed Description of the Invention

The subject invention pertains to the identification of surface-exposed proteins of B. bovis merozoites. The proteins of the invention have sizes of 16, 25, 37, 42, 44, 55, 60, 85, 98, 125, 145, 225, and 250 kDa. The evidence that the proteins are surface exposed includes: (i) monoclonal antibody binding of live merozoites, (ii) labeling by surface iodination, and (iii) sensitivity to mild trypsinization.

We have identified B. bovis merozoite proteins that, by virtue of their surface location and their reactivity with immune bovine sera, are candidates for subunit vaccines. Among the numerous proteins recognized by immune bovine sera, six proteins (37, 42, 55, 85, 125, and 145 kDa) appeared to be relatively immunodominant.

The 145 kDa protein was of parasite origin, but its location on the membrane surface was not directly apparent. This protein may have a small portion exposed at the surface of the merozoite that is sensitive to mild trypsinization but the epitope recognized by the monoclonal antibody located internally.

Immunoprecipitation of radiolabeled antigens with bovine antisera indicated that many Babesia bovis merozoite proteins contain isolate-common epitopes, while at least 8 B. bovis proteins contain species-cross-reactive epitopes. The amino acid sequence of three of the immunogenic proteins from B. bovis (42 kDa) have been determined. Amino acid sequences which deviate in insignificant ways from the disclosed amino acid sequences fall within the scope of the subject invention so long as the antigenic properties of the protein are not altered. Thus, the subject invention includes mutants and fragments of the amino acid sequences depicted herein which do not alter the protein secondary structure, or if the structure is altered, the antigenic activity is retained. In particular, it should be understood that conservative substitutions of amino acids may be made. For example, amino acids may be placed in the following classes: basic, hydrophobic, acidic, polar, and amide. Substitutions whereby an amino acid of one class is replaced with another amino

acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the antigenic activity of the compound.

The ability of antibodies against heterologous geographic isolates to immunoprecipitate proteins from the Mexico B. bovis isolate indicates the conservation of at least one and probably more epitopes between proteins from the heterologous isolates. The conservation of these epitopes is extensive, as many Mexico B. bovis isolate proteins were precipitated by antisera against a different geographic isolate (Honduras). The 42,000 molecular weight Mexico B. bovis protein was precipitated by all five of the undiluted and three of the diluted Honduras antisera.

Among the highly immunogenic B. bovis proteins, only one (the 42,000 molecular weight protein) is both isolate common and species specific. This protein can be used as an antigen for species-specific, antibody-based diagnosis.

Monoclonal antibodies (MoAbs) were generated against surface-exposed proteins on merozoites of B. bovis. A genomic library constructed in the lambda-gt11 expression vector was screened with MoAbs for identification of clones expressing recombinant surface proteins. Four recombinant clones were identified.

Southern blot analyses confirmed the parasite-specificity of the cloned inserts. Western blot analyses demonstrated that recombinant protein production in these clones is IPTG-induced and that the recombinant molecules exist as beta-galactosidase fusion proteins.

Additionally, recombinant proteins, partially purified by affinity column chromatography and gel filtration chromatography, reacted with specific MoAbs in Western blot assay indicating that the integrity of the epitopes is retained during purification. Calves immunized with these partially purified recombinant proteins developed titers of between 10^{-2} and 10^{-5} as evidenced by IFA-live. Immune sera from these animals immunoprecipitated metabolically-radiolabeled merozoite proteins confirming that determinants found on native proteins are expressed by the clones.

DNA probe candidates were also identified using the lambda-gt11 genomic library of B. bovis. Two DNA sequences, designated lambda-Bo6 and lambda-Bo25, hybridized to Babesia DNA but not to bovine DNA. Bo6 detected Mexico and Australia isolates of B. bovis as well as B. bigemina DNA. Lambda-Bo25 demonstrated greater specificity; it did not hybridize detectably to B. bigemina DNA and showed greater sensitivity for Mexico isolates of B. bovis than for Australia isolates.

Thus, lambda-Bo6 is a good candidate for detecting Babesia infections in cattle and ticks, and lambda-Bo25 can be used to distinguish geographic isolates of B. bovis.

Materials and Methods

Strain of B. bovis, Stabilate Preparation, Cryopreservation, and In Vitro Cultures. The strain of B. bovis used in experiments outlined herein was originally isolated from a Boophilus microplus tick-induced infection in Mexico by Dr. R.D. Smith, University of Illinois at Urbana (Goff, W.L. and C.E. Yunker [1986] Exp. Parasitol. 62:202-210). The cloned line was derived from the Mexico isolate by limiting dilution cloning as previously described by Rodriguez et al. (Rodriguez, S.D., G.M. Buening, T.J. Green, and C.A. Carson [1986] Infect. Immun. 42:15-18). The parasites have been maintained in our laboratory by either repeated passages in splenectomized Holstein-Freisian bull calves or in vitro cultivation. Cryopreservation of stabilates of B. bovis-infected erythrocytes obtained from infected calves and the preparation of partially purified merozoites from thawed stabilates has been described (Palmer, D.A., G.M. Buening, and C.A. Carson [1982] Parasitol 84:567; McElwain, T.F., L.E. Perryman, W.C. Davis, and T.C. McGuire [1987] J. Immunol. 138:2298-2304). Continuous in vitro cultivation of B. bovis was performed using a modification of the microaerophilous stationary phase (MASP) culturing system (Goff and Yunker, 1986). Viability of merozoites obtained from either frozen stabilates or in vitro cultivation was confirmed by 6-CFDA assay (McElwain et al., 1987) prior to their use in experiments or immunizations.

Isolation of Merozoites. Merozoites were harvested from cultures after the relative percentage of parasitized erythrocytes was increased by sequential reduction of the concentration of erythrocytes (Goff and Yunker, 1986). For collection of merozoites, the contents of flasks containing >15% parasitized erythrocytes were centrifuged at 400 x g for 10 min at 4°C. The supernatant was centrifuged at 3,000 x g for 15 min at 4°C to pellet the merozoites. The merozoites were suspended in Puck saline-glucose (saline-G), and 2 ml was overlaid on 10 ml of a preformed continuous gradient of 65% Percoll-35% Puck saline-G. The gradient was centrifuged in a swinging bucket rotor at 3,000 x g for 20 min at 4°C. The merozoites were isolated from a band with an approximate density of 1.069 g/ml between erythrocyte ghosts at the Percoll-Puck saline-G interface and the residual intact erythrocyte pellet. The merozoites were washed once in 0.15 M NaCl containing 0.01 M sodium citrate (CS), suspended in CS, and stored on ice until used (within 2 to 4 hr).

Purification, Quantitation and Viability Estimation of Merozoites. An equal volume of the isolated merozoite suspension was mixed with 6-carboxy fluorescein diacetate (6-CFDA; final concentration in CS, 10 ug/ml; Calbiochem-Behring, La Jolla, CA) (McElwain et al., 1987). The mixture was incubated at room temperature for 20 min, followed by centrifugation at 1,000 x g for 10 min and was then suspended in phosphate-buffered saline (PBS; 0.15 M, pH 7.2) for counting on a hemacytometer. The sample was examined with phase microscopy and epifluorescence with a 40X oil objective and fluorescein filter (450 to 520 nm). Viability was assessed as the percentage of total merozoites emitting fluorescence.

Preparation of Immune Bovine Sera. Two spleen-intact Holstein-Freisian steers, 14 months of age and indirect fluorescent-antibody test negative for B. bovis, B. bigemina, and Anaplasma marginale, were inoculated intravenously with approximately 6×10^8 B. bovis-infected erythrocytes from the same blood stabilate used to initiate in vitro cultures. On day 9 postinoculation each steer developed detectable parasitemia and a febrile response which persisted through day 13 postinoculation. Antibody specific for B. bovis was detected with the indirect

fluorescent-antibody test on day 10 postinoculation. The steers were challenge inoculated as before on days 48 and 80 postinoculation, and although the animals did not develop a fever or parasitemia, the antibody titer increased after each challenge. Sera were collected and stored at -70°C after the final challenge, when the indirect fluorescent-antibody test titer was 1:10,000.

In addition, the cloned line was passed through a splenectomized calf whose blood at peak parasitemia was used to infect five 4-5 month old Holstein steers (5×10^7 infected erythrocytes each) and to initiate in vitro cultures. The cattle were reinfected at 23 days post infection (DPI) with 10^8 infected erythrocytes (iRBC) from another splenectomized calf and at 77 and 99 DPI with 10^8 iRBC from culture. At day 127, the five cattle and three weight-matched, previously uninfected controls were infected with 10^9 iRBC from culture. The packed cell volume (PCV) of all animals was monitored daily.

Immunofluorescence of Live Merozoites. Viable merozoites were collected as described above and reacted with the various antibodies by a previously described technique (McElwain et al. [1987], supra). The MoAb-containing ascites fluids were diluted 1/10 (40 ug/ml) in PBS. Immune bovine sera were diluted 1/10 in PBS. An equal volume of each antibody preparation was added to 100 ul of a merozoite suspension and incubated on ice for 1 hr. Each sample was centrifuged at 3,000 x g for 10 min, and the merozoites were washed twice in cold PBS. The samples were then suspended in the appropriate rhodamine-conjugated second antibody (1/40 dilution in PBS) (Kirkegaard and Perry, Inc., Gaithersburg, MD) and incubated on ice for 1 hr. After being washed, the merozoites were suspended in 6-CFDA and incubated for 20 min at room temperature. The merozoites were then centrifuged at 3,000 x g for 10 min at 4°C, suspended in 50 ul of PBS, and examined in wet mounts with appropriate filters for rhodamine (antibody binding) and fluorescein (6-CFDA viability) (546 to 590 nm and 450 to 520 nm, respectively).

Surface Radioiodination. Purity of the gradient separated merozoites was also examined by direct light microscopy of Giemsa stained smears and by transmission electron microscopy of selected samples fixed in 2% v/v glutaraldehyde

in 0.1 M potassium phosphate buffer containing 1% w/v sucrose. Parasites were often arranged in clumps and mixed with very rare erythrocyte ghosts (<0.1%). Merozoites were surface radioiodinated by a previously described lactoperoxidase catalyzed method (Palmer, G.H., and T.C. McGuire [1984] J. Immunol. 133:1010-1015).

Donor erythrocytes (nRBC) from uninfected control cultures were collected, washed three times in PBS, and radiolabeled identically. An equivalent number of nRBC ghosts were prepared by lysing washed uninfected cells from control cultures by freeze/thaw in liquid nitrogen. Ghosts were washed free of hemoglobin in PBS by centrifugation at 35,000 x g, 20 min, 4°C and discarding the supernatant until it was clear. The final pellet was resuspended in PBS for radioiodination by lactoperoxidase.

Metabolic Radiolabeling of Merozoites. Metabolically radiolabeled parasite proteins from calf-derived merozoites were prepared for use in immunoprecipitation experiments according to the methods of McElwain et al. (1987, *supra*) except that cultures containing 100 uCi of [³⁵S]-methionine (³⁵S-Met; New England Nuclear, Boston, MA) per 3 x 10⁹ erythrocytes were incubated at 37°C for 8-9 hr in a Forma Scientific water jacketed incubator instead of a candle jar. Parasites cultivated in vitro were metabolically radiolabeled using normal growth medium (Goff and Yunker, 1986) or D,L-methionine-free medium, addition of 20-400 uCi/ml ³⁵S-Met, ³H-myristic acid, or ³H-glucosamine, and incubation of cultures for 12-20 hr at 37°C. Erythrocytes containing radiolabeled merozoites were solubilized in lysis buffer, TCA-precipitable radioactive counts were determined by a filter paper technique (New England Nuclear), and samples were frozen at -70°C until used.

Phase Separation in TRITONTMX-114. Washed iRBC's from ³⁵S-methionine labeled cultures were lysed in 10 mM Tris, 154 mM NaCl pH 7.4, 1% (v/v) TRITONTMX-114, 1 mM phenylmethylsulfonyl fluoride (PMSF) at 0-4°C and frozen at -20°C. For protein separation, the antigen extract was first processed as described for immunoprecipitation. 10⁷ protein bound counts per minute (CPM) in a volume of 2.0 ml was laid over a 2.0 ml cushion of 6% (w/v) sucrose, 10 mM

Tris, 154 mM NaCl, 1 mM PMSF in a 15 ml conical tube (Bordier, C. [1981] Exp. Parasitol. 20:125-129). The tube was incubated at 37°C for 5 min to allow clouding of the protein extract and then centrifuged at 750 x g at room temperature for 5 min in a swinging bucket rotor. The detergent phase was seen as a thick, oily 100-200 ul pellet and the overlying aqueous phase and sucrose cushion were each removed to separate tubes. The phase separation was repeated twice by adding 200 ul of 15% (v/v) TRITON™X-100 in 10 mM Tris, 154 mM NaCl to the aqueous phase, dissolving the detergent on ice, and re-extracting at 37°C over the same sucrose cushion. The three detergent phases resulting from centrifugation were mixed with 10 mM Tris, 154 mM NaCl at 0-4°C, combined, and TCA-precipitable radioactivity counted along with the aqueous phase.

Immunoprecipitation. Immune sera were used either unadsorbed or adsorbed three times with an equal volume of packed intact nRBC's and three times with an equal volume of nRBC ghosts. Radiolabeled B. bovis or bacterial lysate was processed as described previously (Palmer and McGuire [1984], supra) and incubated overnight at 4°C with 15 ul of bovine serum or 15 ul of serum diluted in Veronal buffered saline (VBS) pH 7.4, 1% (v/v) Nonidet P-40 (NP-40). 150 ul of 10% (v/v) formalinized Protein G-bearing Streptococcus (Omnisorb, Calbiochem, San Diego, CA) in VBS pH 7.4, 1% (v/v) NP-40, 0.1% (w/v) gelatin was added and incubated for 2 hr at 4°C (Akerstrom, B., T. Brodin, K. Reis, and L. Bjorck [1985] J. Immunol. 135:2589-2592). The precipitates were washed twice with VBS, 1% (v/v) NP-40; four times with VBS, 2 M NaCl, 1% (v/v) NP-40, 10 mM ethylenediaminetetraacetic acid (EDTA); and twice more with VBS, 1% (v/v) NP-40. Alternatively, 5 ul of bovine serum, 10 ul of rabbit serum, or 5 ug monoclonal antibody were incubated for 30 minutes at 4°C with radiolabeled lysate. Rabbit anti-bovine or rabbit anti-mouse immunoglobulin sera were added and incubated for 30 minutes at 4°C, followed by 10% v/v protein-A-bearing Staphylococcal aureus for 30 minutes at 4°C. Immune complexes were washed seven times with TEN buffer (20 mM Tris-HCl, 5 mM EDTA, 0.1 mM NaCl, 15 mM NaN₃, pH 7.6) containing Nonidet P-40, and for the second through fifth washes, 2 M NaCl, by

centrifuging at 1250 x g. The precipitated protein was eluted by a described method and either frozen at -20°C or loaded directly onto a polyacrylamide gel (Palmer and McGuire [1984], supra).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
5 of Immunoprecipitates and Autoradiography. Immunoprecipitates were electrophoresed under reducing conditions in 7.5 to 17.5% continuous gradient polyacrylamide gels (Takac, B. [1979] In: Immunological Methods. T. Lefkowitz and B. Persin, eds., p. 81, Academic Press, New York). ¹⁴C-labeled protein standards used for molecular weight determination were myosin, 200 kDa; 10 phosphorylase b, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; and lysozyme, 14.3 kDa (Amersham Corp., Arlington Heights, IL). For ¹²⁵I-labeled antigens, gels were fixed in 30% (v/v) methanol, 10% (v/v) acetic acid, vacuum dried, and exposed to Kodak XAR-2 X-ray film with an intensifying screen at -70°C. For ³H and ³⁵S-labeled immunoprecipitates, gels fixed 15 in 30% (v/v) methanol, 10% (v/v) acetic acid, 10% (w/v) trichloroacetic acid (TCA) were impregnated with En³Hance (New England Nuclear Corp., Boston, MA) prior to drying and exposure to X-ray film at -70°C (Palmer and McGuire, 1984).

Immunoblotting. Immunoblotting of merozoite and recombinant lysogen proteins using monoclonal antibodies was performed as outlined (McElwain et al., 20 1987) using standard procedures (Towbin, H. and J. Gordon [1984] J. Immunol. Methods 72:313-340). Parasite antigen for immunoblotting was prepared from MASP culture flasks with approximately 25% parasitemia. Briefly iRBC's and nRBC controls were collected, washed two times in cold Puck's saline-G and two times in cold PBS, resuspended in PBS, counted, and frozen at -20°C. To remove 25 hemoglobin from lysed cells, the samples were thawed and washed in cold PBS (43,000 x g, 20 min, 4°C) until the discarded supernatant was clear. The final pellet was extracted in lysis buffer, processed identically to radiolabeled antigen for immunoprecipitation, and aliquoted for freezing at -20°C. A volume corresponding to 2.5 x 10⁷ iRBC's or an equivalent total number of nRBC's (10⁸) was mixed with 30 3X SDS-PAGE sample buffer, boiled for 3 minutes, electrophoresed in a 7.5 to

17.5% continuous gradient polyacrylamide gel, and then electrophoretically transferred overnight to a nitrocellulose membrane (Towbin and Gordon, 1984). Immunoblotting using bovine antisera was performed as follows: The nitrocellulose was washed three times quickly in VBS pH 7.4, 0.25% (v/v) TWEENTM20, 0.25% (w/v) gelatin (blocking buffer), incubated 4-6 hr in blocking buffer, cut into strips, and each strip reacted overnight at room temperature with immune serum diluted in blocking buffer. The nitrocellulose strips were then washed three times in blocking buffer and two times in VBS 0.1% (w/v) gelatin prior to incubation for 2 hours at room temperature with ¹²⁵I-Protein G (Amersham Corp.) in VBS pH 7.4, 0.1% (w/v) gelatin (Akerstrom, B., T. Brodin, K. Reis, and L. Bjorck [1985] J. Immunol. 135:2589-2592). The strips were washed twice with VBS 0.1% (w/v) gelatin and four times with 1 M NaCl, 10 mM EDTA, 0.25% (v/v) TWEENTM20. They were then air-dried, taped to cardboard, and exposed to X-ray film with an intensifying screen at -70°C.

Dot Blot Immunoassay. Because MoAbs were used to screen the lambda-gt11 genomic library for clones expressing recombinant surface proteins, they were first evaluated for their ability to bind native antigen applied to nitrocellulose filters. 6-CFDA-positive merozoites were obtained from frozen blood stabilates, lysed in buffer containing 50 mM Tris, 5 mM EDTA, 5 mM iodoacetamide, 1 mM PMSF, 0.1 mM N-alpha-p-tosyl-L-lysyl chloromethyl ketone (TLCK) and 1% NP-40 (lysis buffer), and frozen at -70°C until use. Aliquots of 1 ul containing either 10⁷, 10⁶ or 10⁵ merozoites were spotted onto nitrocellulose filters and air dried. Corresponding numbers of similarly lysed noninfected bovine erythrocytes were spotted on for control. Nitrocellulose filters with spotted antigen were washed three times (10 min each) in buffer containing 10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% TWEENTM20, and 0.1 mM PMSF (TNTP) then incubated in TNTP with 5% nonfat dry milk for 1 hr to block unbound sites. Filters were washed three times in TNTP plus 5% milk, incubated in the same buffer containing 2 ug/ml of specific surface-binding MoAb (1/2 hr), washed three times, incubated for 1/2 hr in a 1:5000 dilution of rabbit anti-mouse immunoglobulin (prepared in our laboratory) in TNTP plus 5%

milk. After three washes, the filters were incubated for 1/2 hr in TNTP plus 5% milk containing 5×10^6 CPM of ^{125}I -labeled Protein A, washed sequentially with TNTP, TNTP plus 0.1% TRITONTMX, and TNTP, then dried and examined by autoradiography.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 - Generation of Monoclonal Antibodies Against Surface Epitopes

Partially purified merozoites of *B. bovis* obtained from frozen blood stabilates were used to immunize BALB/c mice for hybridoma production. Each mouse received an initial subcutaneous immunization of 10^7 6-CFDA-positive organisms in Freund's complete adjuvant followed by 3-4 subcutaneous immunizations of 10^7 6-CFDA-positive organisms in Freund's incomplete adjuvant at 2-4 week intervals. Mouse serum was titered by IFA-live (Barbet, A.F. and T.C. McGuire [1978] Proc. Natl. Acad. Sci. USA 75:1989-1993) after the last immunization, and mice with high titers ($\geq 1:1000$) received an intravenous booster immunization of 10^6 6-CFDA-positive organisms in sterile PBS. Three days later the mice were killed and their spleen cells fused to SP2/0 myeloma cells using standard procedures (McGuire, T.C., L.E. Perryman, and W.C. Davis [1983] Amer. J. Vet. Res. 44:1284-1288). Hybridoma supernates were screened first for convenience by IFA-fixed (Ross, J.P.J and K.F. Lohr [1968] Res. Vet. Sci. 9:557-562). Positive supernates were then screened by IFA-live using stabilate-derived merozoites in order to identify surface reactive MoAbs (McElwain et al. [1987]). The MoAbs were first screened on fixed infected erythrocyte preparations, and four MoAbs were selected for further evaluation because of their distinctive patterns of fluorescence. These patterns included staining of the merozoite cytoplasm and membrane (BABB35A₄), merozoite membrane (BABB90C₄), and merozoite cytoplasm (BABB93A₁) and a

single, punctate reaction appearing polar in location on merozoites (BABB75). The four MoAbs all retained their original specificities after the hybridoma cells were cloned twice and used to produce ascites fluids.

5 Example 2 - Immunoprecipitation of Surface Radioiodinated and Metabolically Radiolabeled Proteins

Spontaneously released merozoites for surface binding and labeling experiments were isolated on Percoll gradients. A large proportion retained their surface coat and >80% were viable, as determined by 6-CFDA staining. On three
10 occasions, 10^8 of these isolated merozoites were inoculated intravenously into susceptible, splenectomized calves. In each case, infection was achieved with a prepatent period similar to that in calves that received an equivalent number of infected erythrocytes from a blood stabilate. Also, in vitro cultures have been routinely reestablished after introduction of these isolated merozoites.

15 Radioiodinated merozoite preparations were immunoprecipitated with the MoAbs described above to confirm the outer surface or cytoplasmic location of the reactive epitopes. BABB35A₄ precipitated a major protein of 42 kDa and a minor protein of 37 kDa. BABB75 and BABB90C₄ precipitated single proteins of 60 and 85 kDa, respectively.

20 To determine which parasite proteins were recognized by the bovine immune system, we used twofold serial dilutions of immune bovine sera to immunoprecipitate metabolically labeled preparations. Proteins with relative molecular masses ranging from approximately 16 to >200 kDa were recognized by the immune bovine sera. Among the proteins recognized were those identical in
25 molecular mass to those precipitated by BABB35A₄ (42 and 37 kDa), BABB75 (60 kDa), and BABB93A₁ (145 kDa). In addition, proteins of 145, 42, 120 and 75 kDa appeared to be immunodominant, as they were precipitated by immune bovine sera at the greatest dilution tested.

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Example 3 - Further Identification of Merozoite Surface Proteins

Subinoculation of 5×10^7 infected erythrocytes (iRBC) of a cloned *B. bovis* line from a splenectomized calf into 4-5 month old cattle caused a 39% reduction in packed cell volume (PCV) (range 31-47). Calves were re-infected three times with approximately 10^8 iRBC of the cloned isolate and then challenged with 10^9 iRBC in concert with three previously uninfected control animals. Only the initial infection caused a significant reduction in PCV when compared to the PCV during the week prior to each infection. Control cattle in the final challenge experiment experienced a 28% reduction in PCV ($p < \text{or} = 0.0005$ when compared to previously infected cattle; Student's paired t test).

Merozoites spontaneously released from culture and purified on Percoll gradients were 95-100% viable by 6-CFDA staining. In all five animals, immunoprecipitation of surface radioiodinated proteins with immune sera that had been extensively adsorbed against donor erythrocytes (nRBC) and erythrocyte ghosts identified seven dominant surface proteins with relative molecular weights of 250, 125, 98, 85, 55, 42, and 37 kilodaltons. The 250 kDa protein does not enter the resolving gel in a standard 14 cm 7.5-17.5% polyacrylamide gel but is clearly resolved in a 25 cm gel. An eighth protein of 25 kDa is immunoprecipitated by immune sera from two calves. Control immunoprecipitation of identically radioiodinated intact nRBC and nRBC ghosts revealed no specific bands on SDS-PAGE.

Adsorbed immune sera was used to immunoprecipitate ^{35}S -methionine metabolically labeled parasite proteins which were run alongside immunoprecipitated surface-iodinated merozoite proteins in a polyacrylamide gel. The immunoprecipitable ^{35}S antigen profile is identical in all five protected animals. The 125, 98, 85, 55, 42, and 37 kDa antigens comigrate perfectly with metabolically labeled proteins. The 25 kDa surface protein that is not identified by immunoprecipitation of methionine labeled antigen does comigrate with a glycoprotein that is metabolically labeled with ^3H -glucosamine. An ^{35}S -methionine labeled 25 kDa protein can be precipitated from other ^{35}S -antigen preparations.

Example 4 - Immunogenicity

While the reactivity of immune sera against the majority of ^{35}S -labeled proteins can be diluted out, sequential serum dilutions (1:160 - 1:640) selectively precipitate the 125, 55, and 42 kDa proteins that were also surface labeled. Because this method is dependent on the specific radioactivity of labeled proteins, dilute sera was also examined for its ability to react with parasite antigens by immunoblotting. Compared to undiluted serum, immune sera diluted 1:500 recognizes a limited number of blood stage proteins including the 125, 85, 55, and 42 kDa surface antigens. The 42 kDa protein is consistently recognized even at dilutions of greater than or equal to 1:16,000.

Example 5 - Further Characterization of Proteins

The immunodominant 42 kDa merozoite surface protein was further characterized as an integral membrane protein based on its hydrophobic nature in phase separated TRITONTMX-114 solution. By definition, integral membrane proteins have a hydrophobic domain that allows interaction with the hydrophobic core of the lipid bilayer and with non-ionic detergents. Parasite proteins were metabolically labeled in culture with ^{35}S -methionine and solubilized in 1% TRITONTMX-114 at 0-4°C. The antigen preparation was warmed above the detergent's cloud point (20°C) and separated into aqueous and detergent phases by centrifugation. Immunoprecipitation from each phase and the starting solution shows that the 42 kDa antigen partitions into the detergent phase.

In order to better characterize merozoite surface antigens, the parasite was examined for the ability to incorporate ^3H -glucosamine and ^3H -myristic acid into immunoprecipitable proteins. Comigration on a polyacrylamide gel shows that three of the surface labeled proteins (55, 42, and 25 kDa) are glycosylated and the 42 kDa glycoprotein is myristylated.

Example 6 - Further Studies on Immunogenicity

Antiserum C151, which was used for immunoprecipitations, was collected from a spleen-intact cow 60 days after experimental infection with a cryopreserved Mexico isolate blood stablate of B. bovis. B. bovis proteins were metabolically labeled in microaerophilus stationary-phase culture by incubation in methionine-deficient medium for 18 to 24 hr with 10 uCi of [³⁵S]methionine per ml. Antiserum C151 immunoprecipitated homologous Mexico isolate proteins biosynthetically labeled with [³⁵S]methionine with molecular weights ranging from 14,500 to greater than 200,000. Serial dilution of this antiserum resulted in a decrease in the number of proteins recognized. Proteins reactive with serum diluted 1:40 had relative molecular weights of 145,000, 120,000 (doublet), and 42,000, while the 42,000 molecular weight protein was still recognized by serum diluted 1:80.

Example 7 - *B. bovis* Proteins with Isolate-Common Epitopes

Five different antisera obtained from cattle after recovery from acute infection with B. bovis in Honduras were able to immunoprecipitate most of the Mexico isolate B. bovis proteins precipitated by C151 antiserum. The 120,000 and 42,000 molecular weight proteins recognized by 1:40 dilutions of C151 antiserum were also recognized by 1:25 dilutions of the Honduran antisera.

Example 8 - *B. bovis* Proteins with Species-Common Epitopes

Antiserum B85 was collected from a spleen-intact calf 25 days after experimental infection with a cryopreserved Mexico isolate of B. bigemina. This antiserum, which had an indirect fluorescent-antibody titer of 1:1,600 against the Mexico B. bigemina isolate, reacted with the Mexico B. bovis isolate at a titer of 1:64. Antiserum C151 (anti-B. bovis Mexico isolate) had indirect fluorescent-antibody titers of 1:5,120 and 1:640 against B. bovis and B. bigemina, respectively. Antiserum B85 immunoprecipitated eight [³⁵S]methionine-radiolabeled proteins of B. bovis. Four of the eight B. bovis proteins immunoprecipitated by B85 antiserum (120,000, 59,000, 53,000, and 19,000 molecular weight) also had isolate-common

epitopes. In addition, the 120,000 molecular weight protein was one of the proteins recognized by C151 serum antibodies diluted 1:40.

Example 9 - Identification of Monoclonal Antibodies

Using similar techniques, additional MoAbs specific for surface-exposed epitopes on live merozoites were identified. All of the identified MoAbs are listed in Table 1. The MoAbs reacted with the outer surface of culture- or stabilate-derived merozoites in either a punctate (restricted to a discrete region on the merozoite surface) or a homogenous (over the entire surface of the merozoite) pattern when examined by IFA-live.

Table 1. Monoclonal antibodies generated against surface proteins on merozoites of Babesia bovis.

MoAb	Isotype	MW of Reactive Protein (x 10 ⁻³ kd)
23.8.34.24	G ₃	225
BABB75	G _{2b}	60
MBOC79	G ₁	60
23.53.156	G _{2a}	60
23.38.120.8	G ₁	60
23.70.174.83	G ₁	44
BABB35A ₄	G _{2a}	42
23.3.16	G ₁	42
23.10.36	G _{2b}	42
23.28.57.108	G _{2a}	16
BABB90C ₄	G ₁	85
BABB93A ₁	G _{2a}	145

All MoAbs reacted with merozoite antigen in dot immunoassay and allowed detection of specific surface-exposed determinants in preparations of 10⁵ lysed

merozoites. The parasite specificity of these MoAb-reactive determinants was confirmed by immunoprecipitation of metabolically-radiolabeled parasite proteins of M_r 16 kDa, 42 kDa, 44 kDa, 60 kDa, and 225 kDa. These proteins have been designated Bo16, Bo42, Bo44, Bo60, and Bo225, respectively. Bo225 was routinely visualized as a tightly spaced doublet when immunoprecipitated with MoAb 23.8.34.24.

Seven monoclonal antibodies and the Mexico isolate of B. bovis have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852 USA. The cultures have been assigned the following accession numbers by the repository:

<u>Biological Material</u>	<u>Deposit number</u>	<u>Deposit date</u>
MoAb 23.38.120.8	HB 10111	May 2, 1989
MoAb BABB93A ₁	HB 10112	May 2, 1989
MoAb 23.8.34.24	HB 10113	May 2, 1989
MoAb 23.70.174.83	HB 10114	May 2, 1989
MoAb BABB35A ₄	HB 10115	May 2, 1989
MoAb 23.28.57.108	HB 10377	March 7, 1990
MoAb BABB90C ₄	HB10117	May 2, 1989
<u>Babesia bovis</u> , Mexico Isolate	ATCC 40601	May 3, 1989

The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 USC 122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposits should the depository be unable to furnish a sample when requested, due to the condition of the deposit(s). All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

Example 10 - Construction of a Lambda-gt11 Expression Library

Partially purified and viable merozoites free from contaminating bovine leukocytes were obtained from frozen stabilates. Merozoites (1.4×10^8 6-CFDA-positive) were lysed in 10 mM Tris, 1 mM EDTA (TE buffer, pH 7.4) containing 2% SDS, and the suspension was treated with DNase-free RNase A (100 ug/ml) followed by Proteinase K (100 ug/ml). Genomic DNA was isolated from the suspension by sequential phenol, phenol/chloroform, chloroform, and ether extractions followed by ethanol precipitation at $0-4^{\circ}\text{C}$ in the presence of 2 M ammonium acetate. The DNA pellet was washed once with 70% ethanol, lyophilized, resuspended in TE buffer and stored at 4°C . The concentration and purity of the DNA were assessed by spectrophotometry and agarose gel electrophoresis. The DNA was sheared into fragments of between 4-8 kb by repeated passages through a 25 gauge hypodermic needle (Young, R.A., B.R. Bloom, C.M. Grosskinsky, J. Ivanyi, D. Thomas, and R.W. Davis [1985] Proc. Natl. Acad. Sci. USA 82:2583-2587) and the fragments prepared for ligation into the EcoRI site of the lambda-gt11 expression vector (Promega Biotec, Madison, WI).

First, fragments were methylated with EcoRI methylase (Promega Biotec) and blunt-ended using the large fragment of E. coli DNA polymerase I (Klenow

Fragment, Bethesda Research Laboratories, Gaithersburg, MD). EcoRI linkers (BRL, Gaithersburg, MD), end-labeled with ^{32}P transferred from 5'-[gamma- ^{32}P]ATP in a kinase reaction (Huyhn et al., 1985), were ligated to fragment termini using T4 DNA ligase (Bethesda Research Laboratories). Free linkers were separated from fragments with EcoRI termini by size fractionation on a Sephacryl S-400 column (Pharmacia AB, Uppsala, Sweden) following digestion of the reaction mixture with EcoRI endonuclease.

Fractions containing fragments with EcoRI cohesive termini but free of nonligated linkers were pooled, butanol-extracted to reduce volume, extracted twice with ether, ethanol-precipitated, lyophilized, and resuspended in TE buffer. Fragments were then ligated into the EcoRI site of the lambda-gt11 expression vector which resulted in the insertion of parasite DNA into the β -galactosidase structural gene (lacZ) of the bacteriophage (Young, R.A. and R.W. Davis [1983] Proc. Natl. Acad. Sci. USA 80:1194-1198). Ligated DNA was packaged into gamma phage heads (Gigapack Gold Packaging Extract, Stratagene Cloning Systems, San Diego, CA) and the resultant library was amplified in E. coli strain Y1090 as described previously (Young and Davis, 1983). The amplified library was stored in sterile SM buffer (0.1 M NaCl, 8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mM Tris [pH 7.5], 2% gelatin) at 4°C.

Example 11 - Identification of Recombinant Phage Expressing Parasite Surface-Exposed Proteins

Recombinant phage expressing proteins with surface-exposed epitopes were identified by immunoscreening plaques with MoAbs. Enough recombinant phage to give 10^5 plaque forming units (pfu)/150 mm diameter petri dish were used to infect E. coli host Y1090 by incubation at 37°C for 20 min in LB medium. Infected cells were added to LB top agar (55°C) containing 100 ug/ml ampicillin and 10 mM MgCl_2 and plated out on 150 mm diameter LB agar plates. Plates were incubated at 42°C for 4 hr to allow plaque formation without concomitant expression of fusion protein. LacZ-directed gene expression was then switched on by overlaying each

plate with a dry nitrocellulose filter saturated previously with 10 mM IPTG and incubating the plates at 37°C for 8-10 hr. After incubation, nitrocellulose filters with bound proteins were marked, removed from the plates, and processed as described previously for dot blot immunoassay. Single plaques expressing recombinant surface epitopes of interest were identified by autoradiography, picked from plates, and rescreened and picked three more times to insure purity of the recombinant phage, stability of the DNA insert, and reliability of recombinant protein expression. Other hosts, such as Salmonella, can be transformed by suitable procedures well known to those in the art.

Approximately 4.2×10^6 recombinant plaques were screened with MoAbs listed in Table 1. Two recombinant clones (lambda-Bo44-15, lambda-Bo44-16) were identified that express a recombinant protein that reacts with MoAb 23.70.174.83 (anti-Bo44) and two (lambda-Bo220-1 and lambda-Bo220-2) that express a recombinant protein that reacts with MoAb 23.8.34.24 (anti-Bo225). When lambda-Bo44-15 and lambda-Bo44-16 were digested with EcoRI, inserts (Bo44-15, Bo44-16) of 1.25 kb were visualized for each recombinant clone. IPTG-induced lysogen preparations of lambda-Bo44-15 consistently produced stronger signals in dot blot immunoassay than did lambda-Bo44-16, and for this reason, lambda-Bo44-15 was chosen for further analysis and use in immunization trials.

Example 12 - Induction of Recombinant Proteins with IPTG

E. coli host strain Y1089 was lysogenized with lambda-gt11 (control) and each of the recombinant clones using standard procedures (Huyhn, T.V., R.A. Young, and R.W. Davis [1985] "Constructing and Screening cDNA Libraries in lambda-gt10 and lambda-gt11," In: DNA Cloning, Vol. 1: A Practical Approach [Glover, D.M., ed.], pp. 49-78, IRL Press, Washington, D.C.). Lysogenized bacteria were examined by dot blot immunoassay in order to determine the ability of clones to produce recombinant protein after induction with IPTG. Each of the recombinant clones, lambda-gt11-infected E. coli Y1089, and noninfected Y1089 controls were grown at room temperature to $OD_{600}=0.8-1.2$. At this time, an

aliquot was removed, centrifuged, and lysed with lysis buffer (1% NP-40). The remaining cells were heated rapidly to 42-45°C for 20 min, IPTG was added to 10 mM, and the cells were incubated in a shaking incubator at 37°C for 1-2 hr to induce protein expression. After incubation, cell cultures were adjusted by addition of LB medium to their OD₆₀₀ prior to addition of IPTG. At this time, an aliquot was removed, centrifuged, and the pellet lysed in lysis buffer. Three ul aliquots of pre- and post-induced bacterial lysates were spotted onto nitrocellulose in triplicate and probed with MoAbs specific for surface proteins or an irrelevant MoAb control (CAEV 4A1) in dot blot immunoassays. Crude lysates of bacteria producing recombinant protein were prepared and stored at -20°C or -70°C until use.

Dot blot and Western blot analysis of lysates of bacteria lysogenized with lambda-Bo44-15 confirmed the inducibility of rBo44-15 with IPTG and its expression as a β -galactosidase fusion protein. rBo44-15 was visible as a doublet of M_r 150 kDa and 165 kDa in Western blots of IPTG-induced preparations probed with MoAb 23.70.174.83. In contrast, rBo44-15 was visible as a single band (165 kDa) in Western blots of identical antigen preparations probed with anti- β -galactosidase. Western blots of lambda-Bo44-15 lysogen preparations probed with an irrelevant IgG₁ control MoAb (5.90.1) showed no reactivity, thus confirming the specificity of reaction observed with MoAb 23.70.174.83.

Example 13 - Purification of Recombinant Proteins

MoAb 23.70.174.83 was purified from ascitic fluid by ammonium sulfate precipitation and DEAE cellulose chromatography and then coupled to Sepharose 4B for immunoaffinity purification of rBo44-15. Solubilized and sonicated rBo44-15 lysogen preparations were applied to the affinity column, the column was washed repeatedly, then adherent recombinant protein was eluted with 0.1 M diethylamine (pH 11.5) containing 0.5% deoxycholate. Elutes were collected directly into 1 M Tris (pH 8.5) then dialyzed against PBS to remove detergents. Aliquots of partially purified protein preparations were boiled for 10 min in SDS sample buffer, subjected to SDS-PAGE, silver stained and examined by Western blot immunoassay

to verify the presence and purity of recombinant protein. Total protein concentration of the preparations was determined using a bicinchoninic acid protein assay (Pierce Chemical Co.).

Partially purified rBo44-15 from affinity column chromatography contained several high M_r proteins ranging from approximately 94 Kd to >165 Kd as well as several lower M_r proteins ranging from 26 Kd to 50 Kd. Western blot analysis of column-purified protein preparations revealed two major bands of reactivity at M_r 165 Kd and 150 Kd that correspond to two major bands present in silver-stained gels. In addition, several bands of lower M_r (26-31 Kd) were observed in Western blots of affinity-purified recombinant protein that were not observed in Western blots of solubilized lysogen preparations prior to affinity purification. These data indicate that immunoaffinity chromatography results in partial degradation of the recombinant molecule without a concomitant loss of integrity of the MoAb-reactive epitope.

Example 14 - Confirmation of the Presence of Surface-Exposed Epitopes on Recombinant Molecules

Five Holstein-Freisian bull calves were each immunized intramuscularly (i.m.) with 100-125 ug of affinity column-purified recombinant protein in Freund's complete adjuvant, followed by four to five additional immunizations at three week intervals of recombinant protein in Freund's incomplete adjuvant. Control calves were immunized similarly with 100 ug ovalbumin. Within one week after the last immunization, calves were bled and their sera heat-inactivated and examined by IFA-live (Goff et al., 1988) to confirm the presence of antibodies to surface-exposed epitopes on merozoites. Hyperimmune bovine serum (KLK C151) and preimmune sera were used as positive and negative controls, respectively. Sera from calves immunized with either rBo44-15 or ovalbumin were used to immunoprecipitate metabolically radiolabeled merozoited proteins (McElwain et al., 1987) in order to verify the specificity of the antibody response.

Antibody titers in serum from calves immunized with partially purified rBo44-15 varied from 10^{-2} to 10^{-5} as evidenced by IFA-live. In contrast, preimmune serum (B452-pre) and serum from ovalbumin-immunized calves showed reactivity with live merozoites at dilutions of 10^{-1} and 10^{-2} , respectively. Antibody in all nondiluted sera or in low serum dilutions (10^{-1} , 10^{-2}) bound to erythrocyte ghosts (infected and noninfected) as well as merozoites within ghosts.

Example 15 - Methods and Materials for Construction of DNA Probe

(a) Parasites and DNA Isolation

Strains of parasites used in this study include a Mexico (M) and Australia (S strain) isolate of B. bovis and a Mexico isolate of B. bigemina. Babesia bovis (M) DNA for both the genomic library preparation and analysis of clones was derived from infected bovine erythrocyte cultures washed three times in phosphate buffered saline (PBS), pH 7.2, followed each time by centrifugation at 400xg. Babesia bovis (S) and B. bigemina DNA was similarly derived from infected calf blood depleted of buffy coats by three washes in PBS. Infected erythrocytes for isolates were differentially lysed in nine volumes of 0.42% NaCl, infected ghosts were pelleted at 400xg, lysed in 5 volumes of 10 mM Tris-HCl (pH 7.5), 10 mM ethylenediaminetetraacetic acid (EDTA), 100 mM NaCl, and 1% sodium dodecyl sulfate (SDS), incubated 16 hr with proteinase K (100 ug/ml), extracted with phenol:chloroform:isoamyl alcohol (24:24:1). DNA in the aqueous phase was spooled after addition of 2 volumes of cold ethanol, spooled DNA was dried and resuspended in 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (TE), treated with RNases A and T1 (15 ug/ml, 15 units/ml, respectively). The solution was reextracted, spooled, dried, and resuspended in TE for use.

To obtain bovine leukocyte DNA, cells in the buffy coat of uninfected blood were processed similar to infected erythrocytes.

(b) Identification and Isolation of Recombinant *B. bovis* DNA

The preparation of the genomic library has been described. Briefly, B. bovis genomic DNA was sheared through a 26 gauge needle to sizes ranging from 4-8

Kb, methylated with EcoRI methylase, EcoRI linkers were added, DNA restricted with EcoRI and separated from digested linkers on sephacryl S-400 (Pharmacia), and DNA ligated into EcoRI digested and dephosphorylated arms of lambda-gt11. Recombinant phage were amplified in Escherichia coli strain Y1089 (Stratagene).

5 DNA from phage plaques was adsorbed onto nitrocellulose and replicate filters were differentially hybridized to nick translated DNA (2×10^6 cpm/ml) from either B. bovis (M) or B. bigemina in 6X SSPE (1X SSPE:150 mM NaCl, 10 mM NaH_2PO_4 , 1 mM EDTA, pH 7.4), 100 ug/ml denatured salmon sperm DNA, and 1% SDS at 65°C for 16 hr. Filters were washed twice in 2X SSPE and 1% SDS at
10 room temperature for 20 min, and twice in 0.1X SSPE and 0.1% SDS at 65°C for 20 min. Dried filters were autoradiographed at -70°C. Recombinant phage hybridizing to B. bovis (M) but not detectably to B. bigemina DNA were purified through 3 rounds of rescreening and isolated for further analysis. Based on characteristics of hybridization to B. bovis (M) genomic DNA, inserts from some
15 recombinants were cloned into plasmid pBS⁺ (Stratagene) to facilitate their analysis.

(c) Southern and Dot Blot Assays

To investigate the genomic organization of candidate probe sequences, restriction fragments were separated electrophoretically on 0.7% agarose gels and transferred to nylon filters. Filters were then hybridized, as described above, in the
20 presence of 10% dextran sulfate, to lambda-gt11 recombinant DNA or preparatively isolated insert DNA that was radioactively labeled. Final wash stringency was either 65°C or 50°C, as indicated.

For dot blot analysis, DNA extracted as described above was spotted onto nylon membranes. Membranes were dried at room temperature, saturated with 0.5
25 M NaOH and 1.5 M NaCl, neutralized in 1 M ammonium acetate and 0.02 M NaOH, rinsed in 6X SSPE, and vacuum-dried at 80°C for 1 hr. Hybridization conditions were similar to those used for Southern blots. Sensitivity of probe sequences was determined for autoradiograms exposed to hybridization filters for approximately 16 hr at -70°C using an intensifying screen.

Example 16 - Candidate DNA Probes for Detecting *Babesia bovis* in Infected Ticks and Cattle

DNA-DNA hybridization assays (DNA probes) are based on the fact that single-stranded DNA will reanneal only with a complementary strand of DNA whose sequence is homologous. DNA probes have been used as a means of detecting various infectious agents, and some are now used routinely in clinical microbiology laboratories. The identification of DNA sequences of *Babesia* spp. makes it possible to create DNA probes for the identification of these species. Therefore, one application of the identification and isolation of genomic sequences which encode babesial antigens is the use of the DNA fragments as DNA probes.

The lambda-gt11 genomic DNA library of *Babesia bovis* was screened to identify DNA probe candidates for direct detection of the parasite in blood or ticks infected with the parasite. Two sequences (lambda-Bo6 and lambda-Bo25) demonstrated superior sensitivity and were analyzed in more detail. The insert size of lambda-Bo6 is 2.75 kilobase pairs (kb). An accurate estimate of the lambda-Bo25 insert was not possible since one of the *Eco*RI insert sites was lost during cloning. However, digestion of lambda-Bo25 with *Eco*RI and *Kpn*I produces a fragment of 2.2 kb which is specific to this clone compared to wild type lambda-gt11. Since the *Kpn*I site in lambda-gt11 occurs approximately 1 kb from the *Eco*RI cloning site, a minimum estimated size of the insert is 1.2 kb.

Inserts from the lambda clones were excised with *Eco*RI (lambda-Bo6) or *Sst*I and *Kpn*I (lambda-Bo25) and cloned into the plasmid BS⁺ (Stratagene), producing the two clones pBo6 and pBo25. Insert fragments preparatively isolated from these plasmid clones were radioactively labeled and used in dot and Southern blot analyses.

Neither sequence hybridized detectably to bovine DNA. pBo6 detected 100 pg of both a Mexico and an Australia isolate of *B. bovis*, but pBo6 also detects 1.0 ng of *B. bigemina* DNA under identical conditions. A unique characteristic of pBo6 is that it hybridizes to a 7.4 kilobase band in uncut genomic DNA of both *B. bovis* and *B. bigemina*. Similarity of restriction enzyme patterns of the pBo6 sequence in

genomic DNA from both geographic isolates suggests that this sequence is well conserved between geographic isolates of B. bovis. Thus, this sequence is a candidate DNA probe for detecting B. bovis infections in cattle and ticks.

pBo25 exhibited no detectable hybridization to bovine or B. bigemina DNA. This sequence detected 100 pg of homologous Mexico isolate DNA, but under identical conditions the sensitivity was reduced to 1 ng for Australia isolate DNA. Restriction enzyme analysis of the pBo25 sequence showed major differences in the number, size, and intensity of bands between the two B. bovis geographic isolates tested. Thus, this sequence can distinguish geographic isolates of B. bovis.

Example 17 - Labeling of DNA Probe

In order to facilitate detection, DNA probes can be labeled in a variety of ways. For example, for biotin labelling, the DNA fragment preparation is adjusted to a concentration of 1 mg/ml (TE) and is mixed with photo-activatable biotin (PAB) at a ratio of 1:3 (DNA:PAB) in a 1.5 ml Eppendorf tube. The tube is placed in an ice bath 10 cm below a 275 W (GE RSM) sunlamp and the DNA + PAB is irradiated for 15 min. The DNA solution is then mixed with an equal volume of 0.1 M Tris-Cl (pH 9.0) and the volume adjusted to 100 ul with H₂O. The unincorporated PAB is extracted from the DNA by the addition of an equal volume of 2-butanol, vortexing, centrifuging briefly, and withdrawing the lower aqueous phase with a Pipetman. The extraction can be repeated to remove any traces of unbound PAB. 3 M NaOAc (pH 5.6) is added to the DNA solution to a final concentration of 0.3 M and the labeled DNA is precipitated by the addition of 3 volumes of ethanol.

After the sample is cooled at -70°C for 15 min, the precipitated DNA is recovered by centrifugation for 10 min. The DNA pellet is dissolved in 10 mM Tris (pH 7.9) and 0.1 mM EDTA. The labeled probe DNA remains stable for 1 year if stored at -20°C.

A non-radioactive method of labeling the DNA probes may be desirable because: 1) the photoactivatable reactions are simple and rapid, 2) the sensitivity

is as high as ^{32}P -labeled probes, 3) the PAB-labeled probes have a long storage life, 4) these probes are relatively inexpensive, and 5) detection of bound probes is by simple colorimetric methods. The radioactive labeling of probes requires the use of ^{32}P , which has a very short half-life (14 days) and is thus unstable and expensive. The use of radioactive probes would be limited because of cost, the dangers of radioactivity, strict requirements for disposal, and the need for licensing.

However, if for some reason the biotin-HRP method of labeling is not acceptable, the DNA fragments can be labeled with [$\gamma\text{-P}$] 32 deoxy CTP by standard nick translation methods.

Example 18 - Description of Recombinant DNA Sequence from *B. bovis* that Encodes an Immunoreactive Epitope Located on the Surface of Merozoites

The cloned insert DNA was excised from the lambda-gt11 vector and recloned into the plasmid BS⁺ (Stratagene), producing the clone pBo44-15. DNA templates for sequencing the insert of pBo44-15 were obtained by creating deletion libraries of this clone using exonuclease III and mungbean nuclease. A different deletion library was obtained starting at each end of the clone, which allowed sequencing of both strands of the insert DNA. The DNA sequence was obtained using the Sanger dideoxy method.

The sequence of Bo44-15 insert DNA is shown in Figure 1. The insert is 1235 base pairs long. The amino acid sequence shown represents the one long open reading frame identified in the sequence. The open reading frame begins at position 1 and encodes a stop codon (TAA) beginning at position 568. This reading frame is in correct register for expression as a fusion protein of β -galactosidase in lambda-gt11, provided the clone is in the correct orientation. A notable feature of this open reading frame is that it would encode a 24 amino acid sequence beginning at AA position 85 which is tandemly repeated beginning at AA position 109. Comparison of these two putative repeats shows only two positions that differ between the repeats as shown below:

30

85 PQRPAETQQTQDSAAPSTPAAPSP 108
 109 PQRPAETQQTQDSTAPGTPAAPSP 132

* *

5 Numbers represent the beginning and ending amino acid position in the open reading frame for each repeat. Letters are the single letter code for amino acids. Asterisks below the aligned repeats indicate amino acid differences between the two repeats.

10 The potential significance of the repeat amino acid sequence is that such repeats are often immunodominant epitopes in surface proteins from a variety of other protozoan parasites, and they induce antibodies that protect against the diseases.

15 The DNA sequences coding for two other of the B. bovis proteins have also been discovered. The DNA sequence for the B. bovis surface proteins of 42 kDA and 60 kDA are shown in Figures 2 and 3, respectively. These sequences, or portions of the sequences, can be used as DNA probes as described in Examples 16 and 17. Also, the proteins produced from cells transformed with these sequences, or portions of these sequences, can be used for vaccines or in the preparation of monoclonal antibodies as described in the examples which follow.

20 The procedure for obtaining these sequences are described below:

25 B. bovis cDNA Expression Library. Erythrocytes from asynchronous B. bovis-infected blood cultures were washed three times in Puck's saline G and stored frozen in liquid nitrogen. Cells were thawed in lysis buffer containing 0.2 M NaCl, 0.2 M Tris-HCl pH 7.5, 1.5 mM MgCl₂, 2% SDS (w/v), and 200 µg/ml Proteinase K and then incubated in lysis buffer at 46°C for two hours (Bradley, J.E., G.A. Bishop, T. St. John, and J.A. Frelinger [1988] Biotechniques 6:114-116). The NaCl concentration of the lysate was adjusted to 0.5 M and poly [A⁺] RNA was isolated by batch adsorption with oligo(dT) cellulose (Bradley et al., supra). DNA eluted with 0.01 M Tris-HCl pH 7.5 was used to prepare a blood stage cDNA library in Lambda ZAP II (Stratagene, La Jolla, CA, USA) (Short, J.M., J.M. Fernandez, J.A.

30

Sorge, and W.D. Huse [1988] Nucl. Acids Res. 16:7583-7600) by a modified Gubler and Hoffman method using EcoRI adapters (Pharmacia LKB, Piscataway, NJ, USA) (Gubler, U., and B. Hoffman [1983] Gene 25:263-269). The cloned insert in plaque purified lambda phage was subcloned into Bluescript SK(-) phagemid using the in vivo excision capabilities of Lambda ZAP II (Short et al., supra).

Immunoscreening. Plaque lifts ont isopropyl thiogalacto-pyranoside soaked nitrocellulose were screened using monospecific rabbit anti-Bv42 antisera (R-914) followed by ¹²⁵I-Protein A and autoradiography, or with anti-Bv60 monoclonal antibody (23.38.120.8) followed by rabbit anti-murine immunoglobulin, ¹²⁵I-Protein A and autoradiography (Young, R.A., and R.w. Davis [1983] Proc. Natl. Acad. Sci. USA 80:1194-1198; Reducker, D.W., D.P. Jasmer, W.L. Goff, L.E. Perryman, W.C. Davis, and T.C. McGuire [1989] Mol. Biochem. Parasitol. 35:239-248). Rabbit R-914 had been immunized with native Bv42 protein immunoaffinity purified using BABB35A₄, a previously described monoclonal antibody (Goff, W.L., W.C. Davis, G.H. Palmer, T.F. McElwain, W.C. Johnson, J.F. Bailey, and T.C. McGuire [1988] Infect. Immun. 56:2363-2368). Positive Bv42 plaques were tested for reactivity with monoclonal antibodies that recognize a Bv42 surface exposed epitope (Goff et al., supra; Reducker et al., supra) as well as an isotype control monoclonal antibody and normal rabbit serum. Recombinant phagemid excised from positive, plaque purified lambda phage was tested for expression by a similar method using colony lifts from transformed, ampicillin resistant E. coli (XL1-Blue strain) (Young and Davis, supra).

Restriction Enzyme Digestion. Lambda rBv42 phagemid DNA was isolated from bacteria by anion exchange chromatography (Qiagen Inc., Studio City, CA) and restriction enzyme digested by standard methods (Maniatis, T., E.F. Fritsch, and J. Sambrook [1982] Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 545 pp.).

Example 19 - Vaccines

Vaccines may be produced from the polypeptides expressed by the parasites themselves or by cells which have been transformed with DNA fragments from

Babesia. By introducing these polypeptides, along with a pharmacologically suitable vehicle or adjuvant, into the animal host, that host can be induced to generate immunological protection against Babesia. The preparation of such a vaccine composition is within the skill of one trained in the medical and immunological sciences. Vaccines may utilize entire polypeptides or epitopes with immunological activity.

Example 20 - Monoclonal Antibodies

Appropriate mice can be immunized with antigens of, or cells expressing antigens of, Babesia. The antigens used for this immunization can be those which are identified and described in the previous examples. The techniques employed to accomplish this immunization procedure are familiar to those skilled in this art. The spleens can then be removed from the immunized mice and the cells therefrom fused to SP-2 myeloma cells using polyethylene glycol. The desired hybrid cells can then be selected by adding hypoxanthine-aminopterin-thymidine to the medium. The surviving cells can then be tested for antibody production. The testing for antibody production can be accomplished using IFA, ELISA, immunoblot, and/or immunoprecipitation procedures.

Example 21 - Detection of *Babesia* Antigens

The monoclonal antibodies, such as those produced by the procedure just described or those disclosed in Examples 1 and 9, can be used to test for the presence of Babesia antigens in a sample of biological fluid. Other monoclonal antibodies to Babesia antigens can also be used. The testing procedure involves contacting the biological fluid with a composition containing one or more of the monoclonal antibodies. If Babesia antigens are present in the biological fluid, then a reaction will occur and this reaction can be detected and quantified by fluorescence or other means.

Example 22 - Detection of Anti-Babesia Antibodies

Anti-Babesia antibodies can be detected in a fluid sample from a bovine suspected of containing these antibodies by performing ELISA procedures on the clinical samples. Generalized ELISA procedures are well known to those skilled in the art. The ELISA procedures or other simple diagnostic procedures of the subject invention could utilize as antigens, for example, whole cell or cell lysate using recombinant microorganisms which express Babesia antigens.

When the biological sample is contacted with the whole cell or cell lysate microorganisms, this contacting is done under conditions which will promote antigen/antibody immunocomplex formation between antigens expressed by the microorganism and antibodies present in the sample. The resulting immunocomplex can be readily detected utilizing standard labeling procedures.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.



American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone: (301)231-5520 • Telex: 898-055 ATCCNORTH • FAX: 301-770-2587

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Dr. Terry F. McElwain
Department of Veterinary Microbiology and Pathology
Washington State University
Pullman, Washington 99164-7040

Deposited on Behalf of: Dr. Terry F. McElwain

Identification Reference by Depositor:

ATCC Designation

Hybridoma, 23.28.57.108

HB 10377

The deposit was accompanied by: ____ a scientific description ____ a proposed taxonomic description indicated above.

The deposit was received March 7, 1990 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

☒ We will not inform you of requests for the strain.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with a living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested March 9, 1990. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon
Bobbie A. Brandon, Head, ATCC Patent Depository

Date: March 12, 1990

cc: ✓ Roman Saliwanchik
Bruce Clary



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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
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AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Terry F. McElwain, D.V.M., Ph.D.
Washington State University
Department of Veterinary Microbiology and Pathology
Pullman, Washington 99164-7040

Deposited on Behalf of: Terry F. McElwain, D.V.M., Ph.D.

Identification Reference by Depositor:

ATCC Designation

Babesia bovis, Mexico Isolate

40601

The deposit was accompanied by: ____ a scientific description X a proposed taxonomic description indicated above.

The deposit was received May 3, 1989 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

X We will not inform you of requests for the strain.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with a living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested February 2, 1990. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon

Bobbie A. Brandon, Head, ATCC Patent Depository

Date: February 8, 1990

cc: ✓ Roman Saliwanchik
Bruce Clary

Claims

1 1. A protein which has a molecular weight selected from the group
2 consisting of: 16, 25, 37, 42, 44, 55, 60, 85, 98, 125, 145, 225, and 250 kDa; and
3 which has one or more of the following characteristics:

- 4 (a) said protein elicits antibodies which bind to the surface of merozoites;
5 (b) said protein elicits an anti-Babesia immunologic response against
6 surface-exposed regions of merozoites; and
7 (c) said protein has merozoite-surface-exposed region(s) that can be
8 labeled with a surface-specific labeling procedure.

1 2. A Babesia merozoite surface protein having a molecular weight selected
2 from the group consisting of 16, 25, 37, 42, 44, 55, 60, 85, 98, 125, 145, 225, and 250
3 kDa.

1 3. The 42 kDa protein, according to claim 2, wherein said protein is a
2 glycosylated integral membrane protein.

1 4. A protein which has the amino acid sequence shown in Figure 1, or an
2 amino acid sequence which is substantially the same, or a fragment of the sequence
3 shown in Figure 1, so long as said substantially same sequence or said fragment
4 retains the biological activity of the sequence shown in Figure 1.

1 5. The protein, according to claim 4, which has the amino acid sequence
2 shown in Figure 1.

1 6. A Babesia bovis protein with species- and isolate-common epitopes
2 capable of eliciting immunologic responses in cattle, said protein having a molecular
3 weight selected from the group consisting of 19, 53, 59, and 120 kDa.

1 7. Monoclonal antibodies specific to a Babesia surface protein, where said
2 Babesia surface protein has a molecular weight selected from the group consisting
3 of: 16, 37, 42, 44, 60, 85, 145, and 225 kDa.

1 8. A monoclonal antibody selected from the group consisting of 23.8.34.24,
2 BABB75, MBOC79, 23.53.156, 23.38.120.8, 23.70.174.83, BABB35A₄, 23.3.16,
3 23.10.36, BABB93A₁, BABB90C₄, and 23.28.57.108.

1 9. A monoclonal antibody reagent useful in determining the presence of
2 pathogens, said reagent containing at least one monoclonal antibody species specific
3 to Babesia.

1 10. A Babesia DNA fragment that expresses a protein that reacts with an
2 antibody selected from the group consisting of: 23.8.34.24, BABB75, MBOC79,
3 23.53.156, 23.38.120.8, 23.70.174.83, BABB35A₄, 23.3.16, 23.10.36, BABB93A₁,
4 BABB90C₄, and 23.28.57.108.

1 11. A Babesia DNA fragment which is selected from the group consisting
2 of lambda-Bo6, lambda-Bo25, lambda-Bo44-15, lambda-Bo44-16, lambda-Bo220-1,
3 and lambda-Bo220-2.

1 12. A DNA fragment comprising a 1.25 kb insert obtained by the digestion
2 of lambda-Bo44-15 or lambda-Bo44-16 with EcoRI.

1 13. The DNA sequence shown in Figure 1, or a DNA sequence which is
2 substantially the same, or a fragment of the sequence shown in Figure 1.

1 14. The DNA sequence shown in Figure 2, or a DNA sequence which is
2 substantially the same, or a fragment of the sequence shown in Figure 2.

1 15. The DNA sequence shown in Figure 3, or a DNA sequence which is
2 substantially the same, or a fragment of the sequence shown in Figure 3.

1 16. A DNA sequence which codes for a polypeptide where said polypeptide
2 has the the amino acid sequence shown in Figure 1.

1 17. A DNA sequence which codes for a polypeptide having the amino acid
2 sequence shown in Figure 2.

1 18. A DNA sequence coding for the polypeptide shown in Figure 4.

1 19. A DNA sequence selected from the group consisting of
2 PQRPAETQQTQDSAAPSTPAAPSP and PQRPAETQQTQDSTAPGTPAAPSP,
3 and immunological equivalents thereof.

1 20. A recombinant microorganism transformed with lambda-Bo6, lambda-
2 Bo25, lambda-Bo220-1; lambda-Bo220-2; lambda-Bo44-15; lambda-Bo44-16; a 1.25
3 kb insert obtained by the digestion of lambda-Bo44-15 or lambda-Bo44-16 with
4 EcoRI; or a DNA sequence which is the same, substantially the same, or a fragment
5 of one of the sequences shown in Figure 1, Figure 2, or Figure 3.

1 21. A recombinant microorganism, according to claim 20, wherein said
2 microorganism is an Escherichia coli or Salmonella spp.

1 22. A recombinant protein produced by a microorganism transformed with
2 lambda-Bo44-15; lambda-Bo44-16; a 1.25 kb insert obtained by the digestion of
3 lambda-Bo44-15 or lambda-Bo44-16 with EcoRI; or a DNA sequence which is the
4 same, substantially the same, or a fragment of, one of the sequences shown in
5 Figure 1, Figure 2, or Figure 3.

1 23. The recombinant protein, according to claim 22, wherein said
2 recombinant protein has the amino acid sequence shown in Figure 1, Figure 2, or
3 Figure 4, or an amino acid sequence which is substantially the same, or a fragment
4 of one of those sequences.

1 24. A recombinant protein, according to claim 22, wherein said recombinant
2 protein elicits an antibody response to babesiosis in calves immunized with said
3 recombinant protein.

1 25. The use of a Babesia bovis protein having a molecular weight selected
2 from the group consisting of 16, 25, 37, 42, 44, 55, 60, 85, 98, 125, 145, 225, and 250
3 kDa, or an immunologic equivalent of said protein, to elicit neutralizing antibodies.

1 26. A vaccine for conferring immunity to babesiosis on a susceptible animal
2 host, said vaccine comprising an immunizing amount of bacterial cells, or products
3 of bacterial cells, where said cells have been transformed with DNA fragments from
4 Babesia that encode a surface protein.

1 27. A vaccine, according to claim 26, wherein said bacterial cells have been
2 transformed with lambda-Bo220-1; lambda-Bo220-2; lambda-Bo44-15; lambda-Bo44-
3 16; a 1.25 kb insert obtained by the digestion of lambda-Bo44-15 or lambda-B-44-16
4 with EcoRI; or a DNA sequence which is the same, substantially the same, or a
5 fragment of one of the sequences shown in Figure 1, Figure 2, or Figure 3.

1 28. A vaccine, according to claim 26, wherein said bacterial cells, or products
2 of bacterial cells, are in combination with an appropriate pharmaceutical carrier.

1 29. A vaccine comprising a protein, or mixture of proteins, where said
2 proteins are Babesia proteins, or immunologic equivalents thereof, where said

3 Babesia proteins have a molecular weight selected from the group consisting of 16,
4 25, 37, 42, 44, 55, 60, 85, 98, 125, 145, 225, and 250 kDa.

1 30. The vaccine, according to claim 29, wherein the protein, or at least one
2 of the proteins, has one of the amino acid sequences shown in Figure 1, Figure 2,
3 or Figure 4, or an amino acid sequence which is substantially the same, or a
4 fragment of one of those sequences.

1 31. A method for vaccinating a susceptible animal host to confer immunity
2 to babesiosis, said method comprising administering an immunizing amount of
3 bacterial cells, or products of bacterial cells, where said cells have been transformed
4 with DNA fragments from Babesia that encode a surface protein.

1 32. A method, according to claim 31, wherein said bacterial cells have been
2 transformed with lambda-Bo220-1; lambda-Bo220-2; lambda-Bo44-15; lambda-Bo44-
3 16; a 1.25 kb insert obtained by the digestion of lambda-Bo44-15 or lambda-B-44-16
4 with EcoRI; or a DNA sequence which is the same, substantially the same, or a
5 fragment of one of the sequences shown in Figure 1, Figure 2, or Figure 3.

1 33. A method, according to claim 31, wherein said bacterial cells, or
2 products of bacterial cells, are in combination with an appropriate pharmaceutical
3 carrier.

1 34. A method for detecting the presence of anti-Babesia antibodies in a
2 clinical sample of material suspected of containing these antibodies, said method
3 comprising the performance of a whole cell or cell lysate ELISA on the said clinical
4 sample, said ELISA is performed using recombinant microorganisms which express
5 Babesia specific antigens.

1 35. A method for detecting the presence of anti-Babesia antibodies in a
2 clinical sample suspected of containing these antibodies, said method comprising

3 (a) contacting the sample with whole cell or cell lysate recombinant
4 microorganisms which express Babesia specific antigens, said
5 contacting done under conditions which will promote specific
6 antigen/antibody immunocomplex formation between antigens
7 expressed by the microorganism and antibodies present in the sample;
8 and

9 (b) detecting immunocomplex formation by means of a label to thereby
10 detect the presence of Babesia antibodies in the sample.

1 36. A method of determining the presence of Babesia in a biological fluid,
2 said method comprising the steps of

- 3 (a) providing an aliquot of the biological fluid to be studied;
4 (b) contacting said aliquot with a measured amount of antibodies species
5 specific to Babesia antigens; and
6 (c) determining whether any reaction with said antibodies occurs.

1 37. A method, according to claim 36, wherein the antibodies have a
2 predetermined fluorescence response to a given optical stimulation.

1 38. A method, according to claim 36, wherein the amount of reaction is
2 determined and the presence of Babesia antigens is quantitatively derived
3 therefrom.

1 39. A method for the detection of evidence of babesiosis in bovine or tick
2 samples comprising tissue or fluid, said method comprising contacting said sample
3 with a DNA probe where said probe comprises labeled single-stranded DNA whose
4 sequence is sufficiently homologous with the DNA of Babesia so that the DNA of
5 the probe specifically binds with the DNA of said Babesia.

1 40. A method for detecting evidence of Babesia bovis and/or Babesia
2 bigemina in a bovine or tick sample comprising tissue or fluid, said method
3 comprising contacting said sample with a DNA probe where said probe comprises
4 the DNA fragment known as lambda-Bo6, or an equivalent thereof.

1 41. A method of detecting evidence of Babesia bovis in a bovine or tick
2 sample comprising tissue or fluid, said method comprising contacting said sample
3 with a DNA probe where said probe comprises the DNA fragment lambda-Bo25,
4 or an equivalent thereof.

1 42. A plasmid selected from the group consisting of pBo6 and pBo25.

1 43. A method, according to claim 39, wherein said probe is labeled by means
2 of radioactive, fluorescent, bioluminescent, or chemiluminescent entities.

1 44. A method, according to claim 39, wherein said probe is labeled by biotin.

1 45. A probe for the detection of babesiosis, said probe comprising labeled
2 single-stranded DNA whose sequence is sufficiently homologous with the DNA of
3 Babesia so that the probe specifically binds with DNA of said Babesia.

1 46. A probe, according to claim 45, wherein said DNA comprises a Babesia
2 DNA which is the same, substantially the same, or a fragment of one of the DNA
3 sequences shown in Figure 1, Figure 2, or Figure 3.

1 47. A probe, according to claim 45, wherein said DNA comprises an insert
2 selected from the group consisting of lambda-Bo6, lambda-Bo25, lambda-Bo44-15,
3 lambda-Bo44-16, lambda-Bo220-1, and lambda-Bo220-2.

10	20	30	40	50	60
\$	\$	\$	\$	\$	\$
GAA TTC AAT GCT TTT CTT AAT GAC AAT CCT CCA CAT ATG TTG ACG AAT GGG AAA GAA AAA					
Glu Phe Asn Ala Phe Leu Asn Asp Asn Pro Pro His Met Leu Thr Asn Gly Lys Glu Lys					
70	80	90	100	110	120
\$	\$	\$	\$	\$	\$
ATG ACT GAA TAT TAC AAA AAA AAT ATA TCC AAG GAA GAT GGT GAG GTA AAG GAT TAC AAA					
Met Thr Glu Tyr Tyr Lys Lys Asn Ile Ser Lys Glu Asp Gly Glu Val Lys Asp Tyr Lys					
130	140	150	160	170	180
\$	\$	\$	\$	\$	\$
ACT ATG GTC AAG TTT TGC AAC GAT TTT CTA GAC AGT AAA TCT CCA TTC ATG AGA CTA TAT					
Thr Met Val Lys Phe Cys Asn Asp Phe Leu Asp Ser Lys Ser Pro Phe Met Arg Leu Tyr					
190	200	210	220	230	240
\$	\$	\$	\$	\$	\$
AAG CAT CTC AAT GAA TAT GAT GAG TTA GTG AAG AAG AAG CCA GCA CAA GAA TCT TCC CCT					
Lys His Leu Asn Glu Tyr Asn Glu Leu Val Lys Lys Lys Pro Ala Gln Glu Ser Ser Pro					
250	260	270	280	290	300
\$	\$	\$	\$	\$	\$
GCT CCT TCA TCC CCG CAA AGA CCT GCT GAA ACC CAA CAA ACT CAG GAT TCA GCT GCA CCT					
Ala Pro Ser Ser Pro Gln Arg Pro Ala Glu Thr Gln Gln Thr Gln Asp Ser Ala Ala Pro					
310	320	330	340	350	360
\$	\$	\$	\$	\$	\$
AAC ACT CCC GCA GCT CCT TCA CCC CCG GAA AGA CCT GCT GAA ACC CAA CAA ACT CAG GAT					
Ser Thr Pro Ala Ala Pro Ser Pro Pro Gln Arg Pro Ala Glu Thr Gln Gln Thr Gln Asp					
370	380	390	400	410	420
\$	\$	\$	\$	\$	\$
TCA ACT GCA CCT GGC ACT CCC GCA GCT CCC TCT CCT CAG GGA CCA ACT GCT GAA AGC CCA					
Ser Thr Ala Pro Gly Thr Pro Ala Ala Pro Ser Pro Gln Gly Pro Thr Ala Glu Ser Pro					
430	440	450	460	470	480
\$	\$	\$	\$	\$	\$
TCC CAA GCT GAC CAC CCA ACC AAA CST ACT CAG ACA CCT GAA GGT AAC CTC CAA GGA CAA					
Ser Gln Ala Asp His Pro Thr Lys Pro Thr Gln Thr Pro Glu Gly Asn Leu Gln Gly Gln					
490	500	510	520	530	540
\$	\$	\$	\$	\$	\$
CAG GGT ACA ACC AAG CCA GCC GGA TCT TCA TTC ACC TAT GGC GGA TTG ACT GTG GCC ACT					
Gln Gly Thr Thr Lys Pro Ala Gly Ser Ser Phe Thr Tyr Gly Gly Leu Thr Val Ala Thr					
550	560	570	580	590	600
\$	\$	\$	\$	\$	\$
CTC TGC TAC TTC GTT CTC TCT GCA TTT TAA TAA CTA ATG GTA GTG ACA CAA TAG TTT TGT					
Leu Cys Tyr Phe Val Leu Ser Ala Phe --- --- Leu Met Val Val Thr Gln --- Phe Cys					
610	620	630	640	650	660
\$	\$	\$	\$	\$	\$
AAA CTC ATG TTT TTT AAC TTT TAA TGA ATG TTA TGT CTA CAG TAT TCG TGG TAC TTC GTC					
Lys Leu Met Phe Phe Asn Phe --- --- Met Leu Cys Leu Gln Tyr Ser Trp Tyr Phe Val					

Figure 1 (continued)

670	680	690	700	710	720
\$	\$	\$	\$	\$	\$
AAT AGT ATA GAC ATA TCT ACA AGC AGA GTA CCA AAA GTT ATA AGC AAT ACT GTG CAC					
Asn Ser Ile Asp Ile Ser Thr Thr Ser Arg Val Pro Lys Val Ile Ser Asn Thr Val His					
730	740	750	760	770	780
\$	\$	\$	\$	\$	\$
GGC ACA AAC TGT TTG TTA TAA CTT TGT CGT TTC ATT TAA CAG AAT ACG TGG TAT ATA TTG					
Gly Thr Asn Cys Leu Leu --- Leu Cys Arg Phe Ile --- Gln Asn Thr Trp Tyr Ile Leu					
790	800	810	820	830	840
\$	\$	\$	\$	\$	\$
GAA TCT CAG GTA GTT CAT ATT CAA TGT CAA TGA TAG CTG ATT AAT TTG TTG TGG ATA TAA					
Glu Ser Gln Val Val His Ile Gln Cys Gln --- --- Leu Ile Asn Leu Leu Trp Ile ---					
850	860	870	880	890	900
\$	\$	\$	\$	\$	\$
CAG TGT GCG GTT GCA TTT CCA TCT CTT GTA ACA TTG ATA TTT GAT GCG ATC AAA CAG AGA					
Gln Cys Ala Ile Ala Phe Pro Ser Leu Val Thr Leu Ile Phe Asp Gly Ile Lys Gln Arg					
910	920	930	940	950	960
\$	\$	\$	\$	\$	\$
TAG CTG CAA TAG GCT AAG CAA ACA AAA GAA TGG CTT TAA TGG AAA TAA AGT TAA GCA TAT					
--- Leu Gln --- Ala Lys Gln Thr Lys Glu Trp Leu --- Trp Lys --- Ser --- Ala Tyr					
970	980	990	1000	1010	1020
\$	\$	\$	\$	\$	\$
ATA TCG TAA AAA TTA AAA TAC TAG CAT ATT GCA GAA TAT AAA CCT GCG TCT GTT TAA TTC					
Ile Ser --- Lys Leu Lys Tyr --- His Ile Ala Glu Tyr Lys Pro Ala Ser Val --- Phe					
1030	1040	1050	1060	1070	1080
\$	\$	\$	\$	\$	\$
TTA ACG AAT AAA AGT GAA ATT GTA ATA CAC ATG TAC ATA CGA TAG ATG GGA TAC ATC CAC					
Leu Thr Asn Lys Ser Glu Ile Val Ile His Met Tyr Ile Arg --- Met Gly Tyr Ile His					
1090	1100	1110	1120	1130	1140
\$	\$	\$	\$	\$	\$
ATG CGA CCA TAT AAC GAT GTG CGA ATT TAG TAT ATT TAT ATC AAA CAT GAT GAT GAA AGG					
Met Arg Pro Tyr Asn Asp Val Arg Ile --- Tyr Ile Tyr Ile Lys His Asp Asp Glu Arg					
1150	1160	1170	1180	1190	1200
\$	\$	\$	\$	\$	\$
AAG GAT TAG AAA ATC AAC ATT TAT AGT GCT CAA TTA TAT ATG AAT GCA ATG TTG TGC TAC					
Lys Asp --- Lys Ile Asn Ile Tyr Ser Ala Gln Leu Tyr Met Asn Ala Met Leu Cys Tyr					
1210	1220	1230			
\$	\$	\$			
ACA GTT TGT CAG AGG CCT GTC AAT GTG TAG AAT TC					
Thr Val Cys Gln Arg Pro Val Asn Val --- Asn					

Translated in frame 1

Translated to 3' end

Sequence printed from base no. 1 to base no. 1235

Sequence numbered beginning with base no. 1

Figure 2

rBv42 NUCLEOTIDE SEQUENCE

```
1          CTTCA ATCGTCCTTC CCGAAGGATC ATTCTACGAT
36  GACATGTCTA AGTTCTACGG TGCTGTTGGA AGTTTCGACC AGACCAAATT
86  GTATAGCGTT CTTTCTGCTA ACTTCAAAGC CGCTAAAATG GATGATCAGA
136 AGGTAAAAGA CACATTCAAA AATTTATACA AAGTCAACGC ATTGATAAAG
186 AACAACTCTA TGATTCGCCC TGATCTATTT AATGCAACTA TTGTTAGCGG
236 TTTTTCAACT AAGAATGACG AGGAAAAATT CAATGCTATA TTTGATTCCA
286 TTAAGGGAAT GTACTATAGA GCTCAACACA TGGACAAATA TTTGAAGTCA
336 CTAAGGTGGA ATACTGATAT TGTTGAGGAA GATCGTGAGA AGGCAGTTGA
386 ATATTTCAAG AAGCATGTTT ATACGGGGGA ACACGTTGTT GACGTCAACG
436 GTATGGCTGG TGTTTGCAAG GAGTTTTTAA GCCCGGCCTC TGATTTCTAC
486 AAAGTTGTTG AGTCTTTTGA TGCGTTTGCA CATGCTAAGG TGCACGCTCA
536 AGTAGGAAAT TTTGTAAAC CTGGAAGTGA CATCGCTCCT CCTAAGGATG
586 TTAGTGATGC ATTAGAAAAG GAATTGCAAG AGCAAAAACC TGCACGAAGT
636 GAGAGCACCG AAGTACCCGC TCCAGGTGAT GCATCTGGCG TCCAACAACC
686 GCCTGCATCA GGAACATCCC CGCAAGGACC TGCTCCGACT ACACCCAGCC
736 CATCTCCAGA GTCCTCAGGA AACCTCCAAG GACAACAGGG TACAACCAAG
786 CCAGCCGGAT CTTCTTTCAC CTATGGCGGA TTGACTGTGG CTACTCTCTG
836 CTACTTCGTT CTCTCTGCAT TTTAAAACT AATGGTAGTG ACACAATAGT
886 TTTGTAAACT CATGTTTTTT AACTTTTAAT ATGTAGTGAA AAA
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TRANSLATION of rBv42

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1          SIVLP EGSFYDDMSK FYGAVGSFDQ TKLYSVLSAN FKAAMDDQK
46  VKDTFKNLYK VNALIKNNPM IRPDLFNATI VSGFSTKNDE EKFNALFDSI
96  KGMYYRAQHM DKYLKSLRWN TDIVEEDREK AVEYFKKHVY TGEHVVDVNG
146 MAGVCKEFLS PASDFYKLVE SFDAFAHAKV HAQVGNFVKP GTDIAPPKDV
196 TDALEKELQE QKPARSESTE VPAPGDASGV QQPPASGTSP QGPAPTTSP
246 SPESGSLQG QGGTTKPAGS SFTYGGLTVA TLCYFVLSAF *
```

Figure 3

1 GACGGATAGT ATTTTACATA TACATTTGTC GACTTTTATA TATAGCAGTG
51 CTATAGACAA ACAATACACA GATTAATCTT TAGATACTAA GTTCATAAT
101 ATTACGBACA TATTGTAGAC AATBAGAAIC ATTAGCBGCG TTGTCEGTTG
151 CCTTTTCTTG GTGTTTTCAC ACCATCTGTC TGCTTTTCGC CACAATCAGA
201 BAGTAGGAAG TCTCECTCCA GCTGANNITG TAGGTGATTT AACCTCCACA
251 TTGSAACAG CTGATACTTT GATBACTCTC CGTGACCACN TGCACACAT
301 TACTAAGGAT ATGAACATG TTTTGAGCAA TGSTCBTGAH CAGATTGTAA
351 ATGATGTTG CTCTAATGCT CCTGAGGACT CCAACTGTCB TCAUGTAGTT
401 AACCAATTAG CTGACCBTTG TGAATGTAC GGAAGCTTTA CGATTGACAA
451 TGTCPAATAT CCSTTGATC AAGAGTACCA ACCTCTATCT GTTCCAAACG
501 GTTACCAGTT GGATCTGCG TTCAGATTGT TCAAGAGAGG TCGATCGAAC
551 CCTGCCAAGA ACAGCETAAA ACGGAPITG TGTCTTTCA GAAATGGAGC
601 GAACCATGGT GATTACCACT ACTCTCTGTC TGCTCTGTTG AAGAGCAATG
651 TTGTGCACGA GGAAGGAACT ACCGATGTTT AATATCTTGT CAACAGGTA
701 CTCTATATGG CTACCATGAA CTACAAGACT TATTTGACAG TAAACGTAT
751 GAGCGCCAAG TTCTTCAACA GATTCAAGCTT CACTACCAAG ATCTTCAATC
801 GTCGTATTAG GCAAACATTG AGTGTATCA TCAGBTGGAA TGTCTCTGAA
851 GATTTTGAAG AAAGGAGCAT CGAACGTATC ACTCAACTTA CTAGCAGTA
901 CSAAGATTAC ATGTTGACCC AGATTCCAC TCTTTCCAAG TTGACACGTC
951 GTTATGCTGA CATGGTGAAG AAGGTTCTGC TCGTAGCTT GACCTCGTAC
1001 GTTGAAGCTC CTTGTACAA AAGATGATA AAGAAITCA GAGACTTTTT
1051 CTCTAAaAAC GTTACCCAAC CTACAAAGAA GTTCATCBAG GATACTAACG
1101 AAGTTACCA AACTATCTG AAGGCCAAAG TTGCTGAGCC CACTAAAGAG
1151 TTTATGCAGG AACTCAGCA AAAAACGAA GGCTATCTGA AAGAGATGT
1201 AGCCGAACCT ACTAAGACTT TTTCAAGGA GGTCTCTCAA GTCACCAAAC
1251 ACTTCTTCA TGAGAACATT GGCACGCCA CCAAGGAGTT TTTAGGGAA
1301 GCTCCCCAAG CCACTAAACA TTCTCTAGAC GAAACATCG GTCAACCAAC
1351 CAAGGAGTTC TTCAGGGAGG CTCTCAAGC CACTAAGCAC TTCTAGGCG
1401 AGATATTGC TCAACCTACT AAGAAITTT TCAAGGATGT CCTCAAGTC
1451 ACCAAGAGGG TTATAACTGA GAACATTGCT CAACCAACTA AGGAGTTCCG
1501 GAGGAGGTT CCTCATGCTA CCATGAAGT CTTGAATGAA AACATTGCTC
1551 AACCTGCCAA GGAAATCATA CATGAGTTTG GTACAGGCGC CAAGAAITTC
1601 ATTTCCGAG CCCATGAAGG TACTAAGCAG TTCTTAAAG AACTGTTGG
1651 CCAACCTACA AAGGATTCC TGAACGAGC TTAGAACT ACTAAGAGC
1701 CATTACCCA TCTGGGTAA TCATCAGAA AAGCCAACT TTATGATGCC
1751 ACGGAAATA CCACTCAGGC TAACGACTCA ACTACTTCCA ACGGTGAAGA
1801 CACCGCCGA TACCTCTGAT GAGATGCTT TATAATGGCA CAAACTCAAC
1851 AAATGATGA TCGTCATCTG ATCCATCGGT TTTCAATATT GTATTGGATG
1901 CAATATCTGA ATGCATATGA TCGACAGTT TCCATCATCG GGTGCCGAAT
1951 CGTAACCTC ATAACACCAT TTTAAGTTAT GCTCGTGCCG

Figure 4

1 MRIISGVVGC LFLVFSHHVS AFRHMQRVGS LAPAEVVGDL TSTLETADTL
51 MTLRDHMHNI TKDMKHVLSN GREQIVNDVC SNAPEDSNCR EVVNNYADRC
101 EMYGCFTIDN VKYPLYQEYQ PLSLFNPFQL DAAFRLFES ASNPAKNSVK
151 REWLRFRNGA NHGDYHYFVT GLLNNNVVHE EGTDDVEYLV NKVLYMATMN
201 YKTYLTVNSM NAKFFNRFSF TTKIFSRRIR QTLSDIIRWN VPEDFEERSI
251 ERITQLTSSY EDYMLTQIPT LSKFARRYAD MVKKVLLSSL TSYVEAPWYK
301 RWIKKFRDFF SKNVTQPTKK FIEDTNEVTK NYLKANVAEP TKKFMQDTHE
351 KTKGYLKENV AEPTKTFFKE APQVTKHFFD ENIGQPTKEF FREAPQATKH
401 FLDENIGQPT KEFFREAPQA TKHFLGENIA QPTKEFFKDV PQVTKKVITE
451 NIAQPTKEFR REVPHATMKV LNENIAQPAK EIIHEFGTGA KNFISAAHEG
501 TKQFLNETVG QPTKEFLNGA LETTKDALHH LGKSSESANL YDATENTTQA
551 NDSTTSNGED TAGYL

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/01812

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁵: A 61 K 39/018, C 07 K 13/00, C 12 P 21/08, C 12 N 15/30,
C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System

Classification Symbols

IPC⁵

C 12 N, A 61 K, C 12 P, G 01 N, C 12 Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	The Journal of Immunology, vol. 138, no.7, 1 April 1987, The American Association of Immunologists, T.F. McElwain et al.: "Antibodies define multiple proteins with epitopes exposed on the surface of live Babesia bigemina merozoites", pages 2298-2304, see the whole article	1,2,4-9
	--	
X	Infection and Immunity, vol. 56, no. 6, June 1988, American Society for Microbiology, T.F. McElwain et al.: "Identification of Babesia bigemina and Babesia bovis merozoite proteins with isolate- and species-common epitopes recognized by antibodies in bovine immune sera", pages 1658-1660, see the whole article	6

* Special categories of cited documents: **

"A" document defining the general state of the art which is not
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"L" document which may throw doubts on priority claim(s) or
which is cited to establish the publication date of another
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invention

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cannot be considered novel or cannot be considered to
involve an inventive step

"Y" document of particular relevance; the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search
29th June 1990

Date of Mailing of this International Search Report

11. 09. 90

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

M. Peis

M. PEIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	--	15,18, 22-24,30, 46
X	Infection and Immunity, vol. 56, no. 9, September 1988, American Society for Microbiology, W.L. Goff et al.: "Identification of Babesia bovis merozoite surface antigens by using immune bovine sera and monoclonal antibodies", pages 2363-2368, see the whole article	1-3,7-9, 29,30
Y	--	14,17, 22-24, 30,46
X	Molecular and Biochemical Parasitology, vol. 22, 1987, Elsevier Science Publishers B.V. (Biomedical Division), A. Gill et al.: "cDNA clone encoding a high molecular weight antigen of Babesia bovis", pages 195-202, see the whole article	1,2,10,11, 20,21, 26-29, 34-41,45, 47
Y	--	12-19, 22-24,30, 42,46
X	Infection and Immunity, vol. 41, no. 1, July 1983, American Society for Microbiology, I.G. Wright et al.: "Babesia bovis: isolation of a protective antigen by using monoclonal antibodies", see pages 244-250, see the whole article	1,2,4,5, 7-9,22-24, 26-30, 34-38
Y	--	12,13,16, 19,22-24, 30,46
	./.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X,O	Journal of Cellular Biochemistry, supplement 13E, 1989, UCLA Symposia on Molecular & Cellular Biology, Abstracts 18th Annual Meetings, 3-24 April 1989, Alan R. Liss, Inc., (New York; US), W. David et al.: "A recombinant Babesia bovis merozoite surface protein elicits antibody in cattle that reacts with live merozoites", page 157, abstract 0616, see the whole article	1,2,4,5, 7-13,16, 19-24,26-30 34-41,43-47
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P,X	Molecular and Biochemical Parasitology, vol. 35, 1989, Elsevier Science Publishers B.V. (Biomedical Division), D.W. Reduker et al.: "A recombinant surface protein of Babesia bovis elicits bovine antibodies that react with live merozoites", page 239-248, see the whole article	1,2,4,5, 7-13,16, 19-24,26-30 34-41,43-47
--		
P,X	Molecular and Biochemical Parasitology, vol. 37, 1989, Elsevier Science Publishers B.V. (Biomedical Division), S.A. Hines et al.: "Molecular characterization of Babesia bovis merozoite surface proteins bearing epitopes immunodominant in protected cattle", pages 1-10, see the whole article	1-3,7-9, 29,30, 34-38

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE :

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers XX because they relate to subject matter not required to be searched by this Authority, namely:

Claims 25, 31 - 33

Pls. see Rule 39.1 (iv) - PCT:

Method for treatment of the human or animal body by therapy or surgery ,
as well as diagnostic method.

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING :

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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